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Rapid Paper

ASYMMETRIC LEAVES2 and Elongator, a Histone Acetyltransferase Complex, Mediate the Establishment of Polarity in Leaves of *Arabidopsis thaliana*

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Leaf primordia are generated around the shoot apical meristem. Mutation of the *ASYMMETRIC LEAVES2* (*AS2*) gene of *Arabidopsis thaliana* results in defects in repression of the meristematic and indeterminate state, establishment of adaxial–abaxial polarity and left–right symmetry in leaves. *AS2* represses transcription of meristem-specific class 1 *KNOX* homeobox genes and of the abaxial-determinant genes *ETTIN/ARF3*, *KANADI2* and *YABBY5*. To clarify the role of AS2 in the establishment of leaf polarity, we isolated mutations that enhanced the polarity defects associated with *as2*. We describe here the *enhancer-of-asymmetric-leaves-two1* (*east1*) mutation, which caused the formation of filamentous leaves with abaxialized epidermis on the *as2-1* background. Levels of transcripts of class 1 *KNOX* and abaxial-determinant genes were markedly higher in *as2-1 east1-1* mutant plants than in the wild-type and corresponding single-mutant plants. *EAST1* encodes the histone acetyltransferase ELONGATA3 (ELO3), a component of the Elongator complex. Genetic analysis, using mutations in genes involved in the biogenesis of a *trans*-acting small interfering RNA (ta-siRNA), revealed that *ELO3* mediated establishment of leaf polarity independently of AS2 and the ta-siRNA-related pathway. Treatment with an inhibitor of histone deacetylases (HDACs) caused additive polarity defects in *as2-1 east1-1* mutant plants, suggesting the operation of an ELO3 pathway, independent of the HDAC pathway, in the determination of polarity. We propose that multiple pathways play important roles in repression of the expression of class 1 *KNOX* and abaxial-determinant genes in the development of the adaxial domain of leaves and, thus, in the establishment of leaf polarity.

Keywords: *Arabidopsis thaliana* • *ASYMMETRIC LEAVES2* (AS2) • *ELONGATA3* (*ELO3*) • Elongator • Histone acetyltransferase • Leaf polarity.

Abbreviations: *AS*, *ASYMMETRIC LEAVES*; GFP, green fluorescent protein; HAT, histone acetyltransferase; HDAC, histone deacetylase; *KNOX*, *knotted*-like homeobox; MS, Murashige and Skoog; RT–PCR, reverse transcription–PCR; ta-siRNA, *trans*-acting small interfering RNA; TSA, trichostatin A; UTR, untranslated region

Introduction

Leaves develop, as lateral organs, from the peripheral zone of the shoot apical meristem. Initially, a group of cells, which might be in a determinate state, is generated along the proximal–distal axis. The subsequent establishment of the adaxial–abaxial axis in early-stage leaf primordia is crucial for further leaf development, as cells proliferate along the medial–lateral axis, and results in the flat and symmetrical shape of leaves (Steeves and Sussex 1989, Hudson 2000, Tsukaya 2006).

The *PHANTASTICA* (*PHAN*) gene of *Antirrhinum majus* is involved in the growth and adaxial–abaxial determination of lateral organs and is required early in the establishment of the proximal–distal axis (Waites and Hudson 1995, Waites et al. 1998). The *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*) and *REVOLUTA* (*REV*) genes, which encode class III homeodomain-leucine zipper (HD-ZIPIII) proteins, determine adaxial cell fate (McConnell and Barton 1998, McConnell et al. 2001, Emery et al. 2003) and are negatively regulated by microRNA165 (miR165) and miR166 (miR165/166) (Bao et al. 2004, Mallory et al. 2004). Members of the *YABBY* (*YAB*) family, such as the

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FILAMENTOUS FLOWER (*FIL*) gene, and of the *KANADI* (*KAN*) family, which are involved in the specification of abaxial cell fate in the leaf lamina, have also been identified (Bowman and Smyth 1999, Sawa et al. 1999, Siegfried et al. 1999, Eshed et al. 2001, Kerstetter et al. 2001, Kumaran et al. 2002, Eshed et al. 2004, Wu et al. 2008, Sarojam et al. 2010). In addition, *ETTIN*/*AUXIN RESPONSE FACTOR3* (*ETT*/*ARF3*) and *ARF4* specify both abaxial cell fate and lateral growth (Pekker et al. 2005). Thus, the mediolateral growth of the lamina is related to the determination of adaxial–abaxial identity (Waites and Hudson 1995, Eshed et al. 2004, Golz et al. 2004). Levels of transcripts of both *ETT*/*ARF3* and *ARF4* are regulated by a set of *trans*-acting small interfering RNAs (ta-siRNAs) that are known as tasiR-ARFs. These tasiR-ARFs are derived from non-coding *TAS3* transcripts, which are initially targeted for cleavage by miR390 and a TAS3-specific small RNA-processing protein, ARGONAUTE7 (AGO7)/ZIPPY. Then the tasiR-ARFs are amplified as a consequence of the activities of the products of *SUPPRESSOR OF GENE SILENCING3* (*SGS3*) and *RNA-DEPENDENT RNA POLYMERASE6* (*RDR6*) and, finally, they are processed by the products of *DICER-LIKE4* (*DCL4*) (Allen et al. 2005, Williams et al. 2005, Xie et al. 2005, Adenot et al. 2006, Faulgren et al. 2006, Hunter et al. 2006, Nagasaki et al. 2007, Nogueira et al. 2007). *AGO7* and *TAS3* are expressed on the adaxial side of leaf primordia, ensuring that *ETT* and *ARF4* transcripts are restricted to the abaxial side of the primordia (Montgomery et al. 2008, Chitwood et al. 2009, Schwab et al. 2009).

The *ASYMMETRIC LEAVES1* (*AS1*) and *AS2* genes of *Arabidopsis thaliana* are involved in the formation of appropriately expanded and flat leaves, with generation of the three structural axes mentioned above (Rédei and Hirono 1964, Tsukaya and Uchimiya 1997, Byrne et al. 2000, Ori et al. 2000, Byrne et al. 2001, Semiarti et al. 2001, Byrne et al. 2002, Iwakawa et al. 2002, Ikezaki et al. 2010). *AS1* encodes a myb domain protein and *AS2* encodes a plant-specific protein with an AS2/LOB domain, and both these gene products are nuclear proteins (Byrne et al. 2000, Iwakawa et al. 2002, Sun et al. 2002, Shuai et al. 2002, Ueno et al. 2007, Matsumura et al. 2009). The two proteins form a complex (Yang et al. 2008, referred to as AS2/AS1 in this report) that is involved in proper axis formation. During leaf development, AS2/AS1 represses both the expression of genes for abaxial determinants (Iwakawa et al. 2007, Takahashi et al. 2008) and the expression of class 1 *KNOTTED*-like homeobox (*KNOX*) genes, such as *BREVIPEDICELLUS* (*BP*), *KNAT2*, *KNAT6* and *SHOOT MERISTEMLESS* (*STM*), which are normally expressed in the shoot apical meristem and its periphery and which appear to function in the maintenance of the indeterminate cell state (Lincoln et al. 1994, Long et al. 1996, Byrne et al. 2000, Ori et al. 2000, Semiarti et al. 2001, Iwakawa et al. 2007, Guo et al. 2008, Ikezaki et al. 2010).

Many mutations, such as mutations in genes for ribosomal proteins, for components of the 26S proteasome, and for biogenesis of tasiR-ARFs, modify or enhance leaf adaxial–abaxial patterning on the *as1-1* or *as2-1* genetic background (Xu et al. 2003, Li et al. 2005, Garcia et al. 2006, Huang et al. 2006, Xu et al. 2006, Yang et al. 2006, Pinon et al. 2008, Yao et al. 2008, Horiguchi et al. 2011, Szakony and Byrne 2011). Mutations in the chromatin-remodeling gene *PICKLE*, the *HIRA* gene for a histone chaperone and the *HDT1* and *HDT2* genes for histone deacetylases (HDACs) also modify the phenotype of *as1*/*as2* mutant plants (Ori et al. 2000, Phelps-Durr et al. 2005, Ueno et al. 2007), indicating that modifications of chromatin play an important role in leaf development. Thus, it appears that multiple pathways regulate leaf development on the *as1* or *as2* background, but the roles of the various genes and the mutual interactions among their products during leaf development are unknown.

In the present study, we identified two enhancers of the abnormal leaf development phenotype associated with *as2*, namely *east1-1* and *east1-2*. Mutations in these enhancers correspond to mutations in the *ELONGATA3* (*ELO3*) gene, which encodes a homolog of Elp3 of yeast and animals. Mutations in *ELO3* increased the adaxial defects of leaves on the *as2* and *as1* backgrounds. The Elongator complex was first identified as a factor that associates with hyperphosphorylated RNA polymerase II in yeast (Otero et al. 1999). Subunits of the Elongator complex, designated Elp1–Elp6, have been purified from yeast, human and plant cultured cells (Wittschieben et al. 1999, Hawkes et al. 2002, Winkler et al. 2002, Nelissen et al. 2010). Previous studies showed that the complex promotes transcription via acetylation of histone H3 and histone H4 (Kristjuhan et al. 2002, Kouskouti and Talianidis 2005, Han et al. 2008, Nelissen et al. 2010), and maintains gene silencing and genome stability during DNA replication in yeast (Li et al. 2009). Mutations in the components of the Elongator complex, encoded by *ELO2*/*ABA-OVERLY SENSITIVE 1* (*ABO1*)/*AtElp1*, *AtElp2*, *ELO3*, *ELO1*/*AtElp4*, *AtElp5*, *AtElp6* and the putative regulator *DEFORMED ROOTS AND LEAVES1* (*DRL1*)/*ELO4* in *A. thaliana*, have pleiotropic effects, delaying growth, inducing the formation of narrow leaves and increasing sensitivity to ABA (Nelissen et al. 2003, Nelissen et al. 2005, Chen et al. 2006, Falcone et al. 2007, Zhou et al. 2009, DeFraia et al. 2010). We propose that the Elongator complex regulates the adaxial–abaxial patterning of leaves, acting in concert with the products of the *AS1* and *AS2* genes in *A. thaliana*.

Results

Identification of genes for enhancers of *as2*-mediated defects in leaf development

In a genetic screening for enhancers and suppressors of the *as2-1* mutation, we identified a number of mutations that modified the phenotype of *as2-1* leaves (**Fig. 1**). However, we failed to isolate any suppressors of the *as2-1* mutation. We analyzed two independently isolated mutants with similar phenotypes in terms of leaf morphology; each had filamentous leaves and trumpet-shaped leaves. Allelism analysis demonstrated that the two mutants had mutations at the same locus. We named the

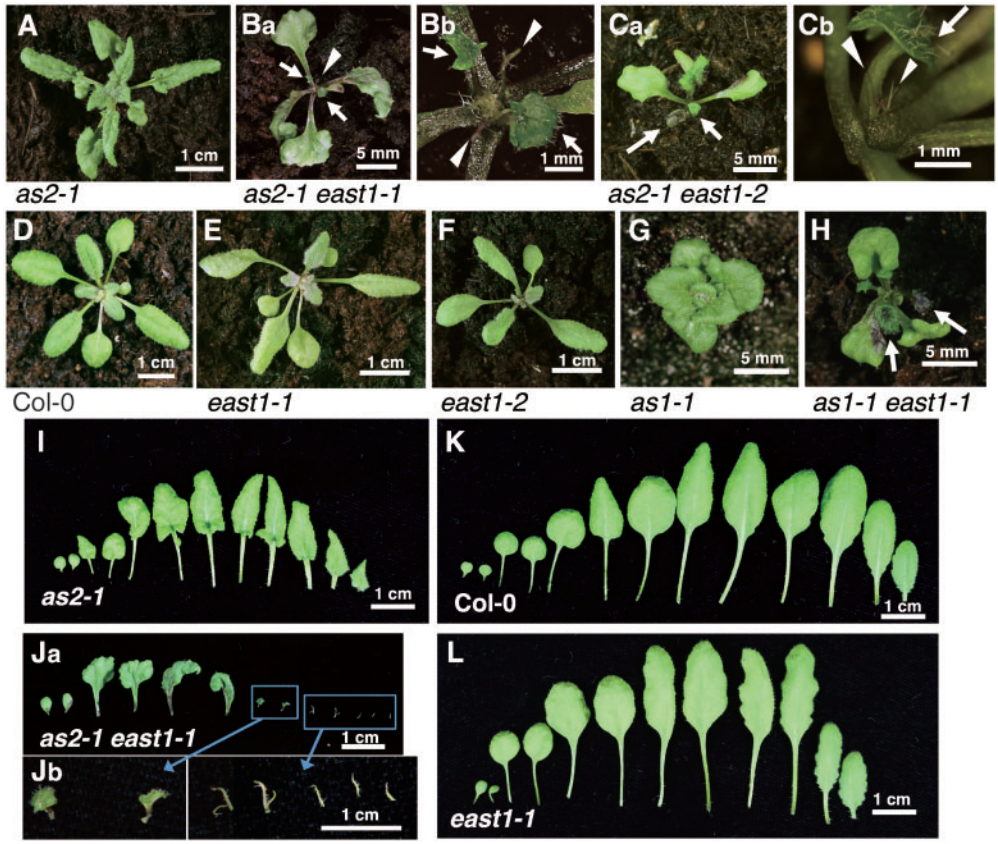

Fig. 1 The *east1-1* and *east1-2* mutations enhance the adaxial abnormality of *as2-1* leaves. (A) Gross morphology of *as2-1*. (Ba) Gross morphology of *as2-1 east1-1*. Arrowheads indicate filamentous leaves. (Bb) Magnified view of Ba, showing filamentous leaves of *as2-1 east1-1*. (Ca) Gross morphology of *as2-1 east1-2*. (Cb) Magnified view of Ca, showing filamentous leaves and trumpet-shaped leaves of *as2-1 east1-2*. (D) Wild-type plant (Col-0). (E) *east1-1*. (F) *east1-2*. (G) *as1-1*. (H) *as1-1 east1-1*. (I–L) Cotyledons and the first to the 10th [or 11th for Col-0 (K)] rosette leaves of *as2-1* (I) and *as2-1 east1-1* (Ja). Magnified view of trumpet and filamentous leaves (Jb) and arrays of Col-0 (K) and *east1-1* (L) leaves. Plants shown in A–H were photographed 21 d (A), 27 d (B–F), 28 d (G) and 42 d (H) after sowing. White arrowheads and arrows indicate filamentous leaves and trumpet-shaped leaves, respectively.

mutations *east1-1* (*enhancer-of-asymmetric-leaves-two1-1*) and *east1-2* (**Fig. 1Ba, Ca**). The *as2-1 east1-1* double mutant developed several small downwardly curled leaves and filamentous leaves during later phases of growth (**Fig. 1Ba, Bb, Ja, Jb**). The *as2-1 east1-2* mutant had more severe defects, with only a few abnormally shaped leaves with greatly reduced lamina, as well as filamentous leaves (**Fig. 1Ca, Cb**), and this double mutant rarely produced flowers and seeds. The phenotypes associated with the two mutant alleles were observed on the *as2-1* genetic background but not on the wild-type background (**Fig. 1Ba, Ca, E, F**, **Table 1**). The *east1-1* single mutant grew slowly and had pale-green and downwardly curled leaves (**Fig. 1E, L**). The *east1-2* mutant grew even more slowly and had smaller leaves than the *east1-1* mutant (**Fig. 1F**). We generated an *as1-1 east1-1* double mutant, which also had trumpet-shaped and filamentous leaves with short petioles (**Fig. 1H**). The efficiency of formation of filamentous leaves on *as1-1 east1-1* plants was, however, lower than that on *as2-1 east1-1* plants (**Table 1**).

Table 1 Frequency of abnormal leaves in single and double mutants

	No. of plants examined	No. and percentage of plants with trumpet-shaped leaves	No. and percentage of plants with filamentous leaves
as2-1	80	0 (0%)	0 (0%)
east1-1	49	0 (0%)	0 (0%)
as2-1 east1-1	65	53 (82%)	62 (95%)
as1-1	88	0 (0%)	0 (0%)
as1-1 east1-1	48	25 (61%)	28 (68%)

Since the *as2-1 east1-2* mutant rarely produced flowers and seeds, we focused on the *east1-1* mutation in subsequent analysis. The development of vein networks is less extensive in cotyledons and leaves of the *as2-1* mutant than in the wild

type (Semiarti et al. 2001). We also examined venation patterns in the cotyledons and first two leaves of *as2-1 east1-1* and corresponding single-mutant plants. The *east1-1* mutation had slight defects in patterns of leaf venation. However, the *as2-1 east1-1* leaves had more severely defective formation of leaf veins than the single *as2-1* and *east1-1* mutant leaves (**Supplementary Fig. S1**).

The *as2-1 east1-1* mutant was defective in the establishment of the adaxial cell fate in leaves

To examine the effect of the *east1-1* mutation on the adaxial–abaxial polarity of leaves, we used a fusion construct with the gene for green fluorescent protein (GFP) under the control of the *FIL* promoter (*FILp:GFP*), which is expressed in abaxial cells of leaf primordia (Watanabe and Okada 2003). When we examined the *as2-1* shoot apex from the adaxial side of leaves, we detected a faint signal due to GFP at the edges of developing young leaves (**Fig. 2A**). We then detected strong signals due to GFP on the abaxial side in sections of leaf primordia (**Fig. 2B**). When we examined the *as2-1 east1-1* shoot apex from the adaxial side of leaves, we detected strong signals at the edges and over the entire surface of serrated leaf blades and petioles (**Fig. 2C**) and on the entire surface of the filamentous leaves at the fourth and fifth positions (**Fig. 2D**).

To evaluate the effects of the *east1-1* mutation on gene expression, we performed real-time reverse transcription–PCR (RT–PCR) using RNA from aerial parts of 14-day-old plantlets (shown in **Fig. 2E–H**) and quantified transcripts of class 1 *KNOX* genes, which are expressed in the shoot apical meristem and at its periphery in wild-type plants; of *HD-ZIP III* genes (*PHB*, *PHV* and *REV*), which specify adaxial cell fate; and of three families of transcription-related genes, namely *YAB* genes, *KAN* genes and *ARF* genes, which separately and redundantly specify abaxial cell fate. As shown in **Fig. 2I**, levels of transcripts of a number of genes that are involved in the establishment of abaxial cell fate were higher in the *as2-1 east1-1* double mutant than in the wild-type and corresponding single-mutant plants. However, levels of *HD-ZIP III* transcripts were not significantly different. These results suggested that the filamentous leaves of *as2-1 east1-1* plants might have accentuated abaxialized characteristics.

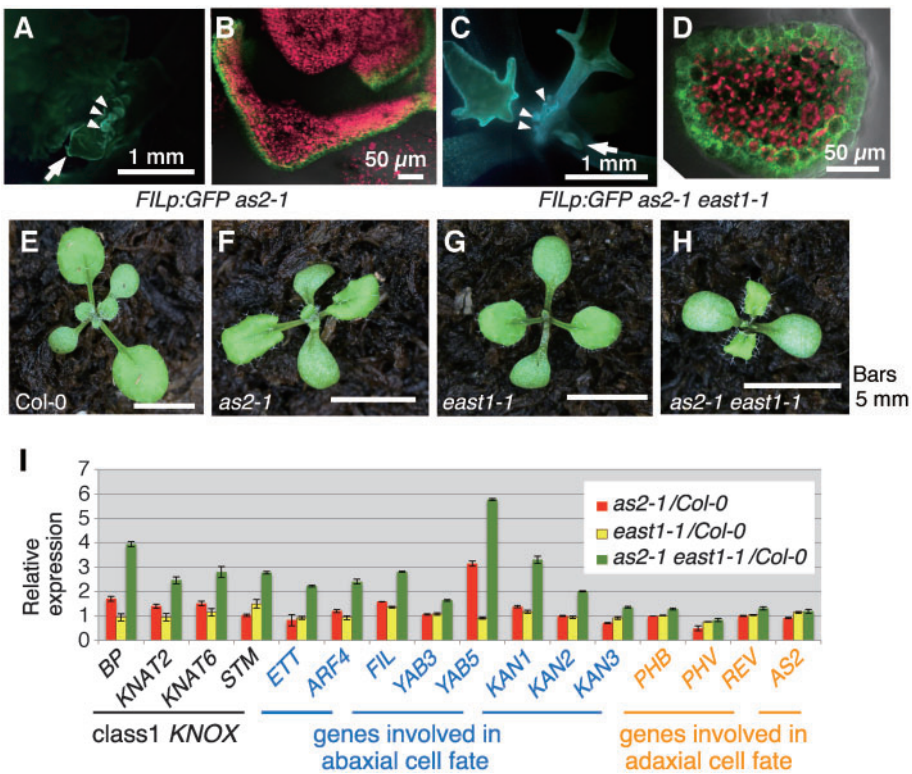

Fig. 2 Filamentous leaves of *as2-1 east1-1* were abaxialized. (A) Signals from GFP in the shoot apex of *as2-1* that harbored the *FIL promoter:GFP* (*FILp:GFP*) construct. Signals due to GFP were observed from the adaxial side. An arrow and arrowheads indicate developing young leaf and flower buds, respectively. Plants shown in A–D were photographed 21 d after sowing. (B) Signals due to GFP (green) and Chl autofluorescence (red) in sections of leaf primordia of *as2-1 FILp:GFP*. (C) Signals due to GFP in the shoot apex of *as2-1 east1-1* that harbored the *FILp:GFP* construct. An arrow and arrowheads indicate developing young leaf and flower buds, respectively. (D) Signals due to GFP in a transverse section of a filamentous leaf in *as2-1 east1-1 FILp:GFP*. (E–H) Plants used for the expression analysis in I: Col-0 (E), *as2-1* (F), *east1-1* (G), *as2-1 east1-1* (H). (I) Expression analysis by quantitative real-time RT–PCR. Levels of expression relative to those in wild-type plants (Col-0) are shown in the histogram. Results were normalized by reference to levels of *ACTIN2* transcripts. Total RNA was extracted from above-ground parts of seedlings 14 d after sowing.

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Accumulation of *KNOX* transcripts was also greater in the *as2-1 east1-1* double mutant than in the wild type, and *as2-1* and *east1-1* single-mutant plants. These results suggest that *as2-1 east1-1* leaf cells are in a more indeterminate and more abaxialized state than the wild-type leaf cells.

The *east1-1* and *east1-2* mutations were mapped to the *ELONGATA3* gene

The locus responsible for the *east1* phenotype was located between 20.1 and 21.7 Mb on chromosome V, as determined from an analysis of 533 F_2 segregants. The site of the *east1-1* mutation was mapped to a 50 kb region of the genomic DNA. Sequence analysis revealed that the *east1-1* and *east1-2* mutations corresponded to one-base and two-base substitutions, respectively, in At5g50320 (gene code of the Arabidopsis Genome Initiative), namely *ELONGATA3* (*ELO3*) (**Fig. 3A**). The ELO3 and ELP3 proteins of other eukaryotes include an N-terminal domain that is conserved in the radical *S*-adenosylmethyltransferase superfamily (radical SAM domain) and a C-terminal GNAT-type histone acetyltransferase (HAT) domain that is related to GCN5 (Wittschieben et al. 1999, Pandey et al. 2002; **Supplementary Fig. S2**).

In the *east1-1* and *east1-2* mutants, levels of the corresponding mRNAs were the same as in the wild-type plants (**Fig. 3C**). However there was one type of *east1-1* mRNA and four types of *east1-2* mRNA (**Fig. 3B**). In the product of *east1-1*, we found that a conserved arginine residue had been changed to a tryptophan residue in the radical SAM domain (**Fig. 3A**, **Supplementary Fig. S2**). The *east1-2* mutant had two independent base substitutions in the *ELO3* gene: one caused replacement of a non-conserved alanine residue by a glycine residue in the radical SAM domain (**Fig. 3A**); the other was a single base change (G to A) at the 5′-splice site of the fourth intron, which resulted in four classes of abnormally spliced transcripts (*east1-2a*, *-2b*, *-2c* and *-2d*; **Fig. 3B, C**). The major transcript in *east1-2* plants (**Fig. 3B, C**), designated *east1-2a*, included the non-spliced fourth intron, which caused the mistranslation of 61 amino acid residues and a stop codon within the radical SAM domain. Minor splicing variants in *east1-2* plants, designated *east1-2b* through *east1-2d*, also included the respective internal stop codons within the radical SAM domain (indicated by asterisks in **Fig. 3B**). In *east1-2b*, the fourth exon lacked 8 bp at the 3′ end and was joined to the fifth exon, with a resulting premature stop codon in the fourth exon. The *east1-2c* transcript included a 12 bp sequence at the 3′ end of the third intron and the 93 bp fragment at the 5′ end of the fourth intron. The *east1-2d* transcript included the 67 bp fragment at the 5′ end of the fourth intron, and a frameshift created a stop codon in the fifth exon. All proteins predicted from the various *east1-2* mutant mRNAs appeared to lack the HAT domain. These results are consistent with the observation that *as2-1 east1-2* plants had more severe defects than *as2-1 east1-1* plants, which generated the full-length protein with a single amino acid substitution in the radical SAM domain.

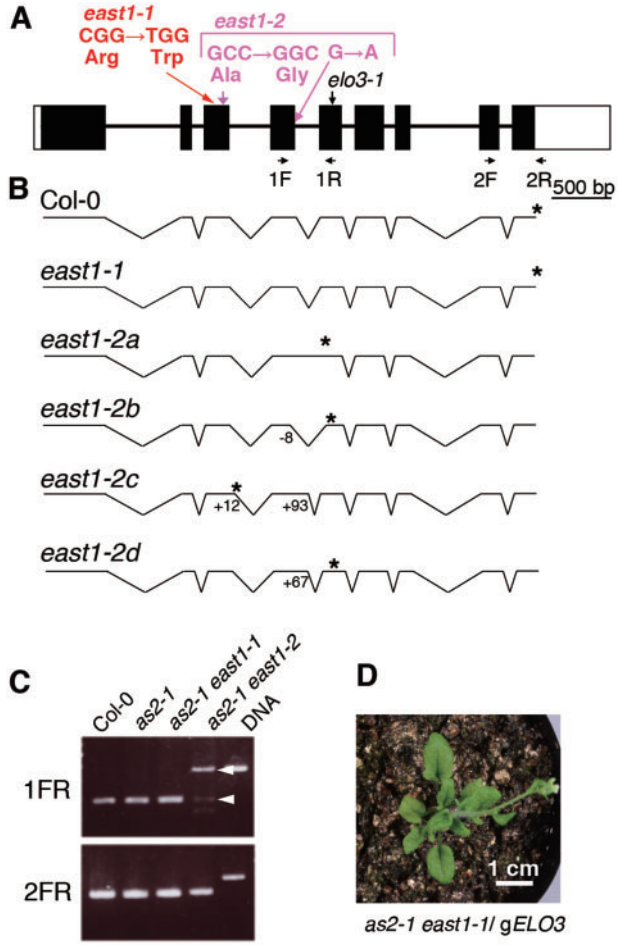

Fig. 3 The *east1-1* and *east1-2* mutations are localized in the *ELONGATA3* gene. (A) Genomic structure of *ELO3* (At5g50320). Black and white boxes indicate the coding region and UTR, respectively. Sites of mutations are indicated by red (*east1-1*) and pink (*east1-2*) arrows. Small black arrows indicate the positions of primers, namely 1F, 1R, 2F and 2R, for RT–PCR. (B) Transcripts from Col-0, *east1-1* and the major transcript (a) and splicing variants (b–d) from *east1-2* are shown. Horizontal lines indicate exons. The fourth intron was not spliced out from *east1-2a* mRNA. The cryptic spliced sites in *east1-2b*, *-2c* and *-2d* are shown with numbers, with + indicating downstream and – indicating upstream of the normal splice site. Asterisks indicate stop codons. (C) RT–PCR analysis of *ELO3* transcripts in Col-0, *as2-1*, *east1-1* and *east1-2*. In the analysis of RNA from *east1-2*, multiple bands (white arrowheads) were detected with primers 1F and 1R. (D) Complementation test with *as2-1 east1-1* and a genomic fragment of the *ELO3* genes (see text for details).

We performed a complementation test using *as2-1 east1-1* double mutants and a 5.6 kb genomic fragment that contained the *ELO3* coding region; a 0.5 kb upstream region, which contained part of the 3′-untranslated region (UTR) of an adjacent gene; and a 1 kb 3′-downstream region. An *as2-1 east1-1* transformant harboring the *ELO3* genomic fragment had the *as2-1* single-mutant phenotype (**Fig. 3D**). T_2 progeny also had a

typical *as2-1* single-mutant phenotype in the presence of the transgene. We concluded that mutations in *ELO3* were responsible for the enhanced mutant phenotype of the *as2-1 east1-1* double mutant plants.

Mutation of the gene for another component of the Elongator complex affected establishment of leaf adaxial–abaxial polarity

To investigate the relationship between AS2 and the Elongator complex, we examined whether other *elongata* mutations might result in enhanced defects in adaxial–abaxial polarity on the *as2-1* background. First, we produced the double mutant *as2-1 elo2-1* with the L*er* genetic background, by crossing *elo2-1* (L*er*) with an *as2-1* plant backcrossed twice with L*er*. The *as2-1 elo2-1* mutant had filamentous leaves (**Fig. 4B**). Secondly, we produced an *as2-1 elo2-3* mutant by crossing *as2-1* (Col-0) with an *elo2-3* plant, which is a T-DNA insertion line with the Col-0 background (Nelissen et al. 2005). The *as2-1 elo2-3* mutants produced trumpet-shaped leaves (**Fig. 4C**). The incomplete effect of *elo2* on the Col-0 background has been described previously (Nelissen et al. 2005).

The *elo4* mutation and the weak allele *drl1*, also caused weakly defective adaxial–abaxial polarity on the *as2-1* background. We produced *as2-1 elo4* plants with the L*er* background, by crossing *elo4* (L*er*) with an *as2-1* plant backcrossed twice with L*er*. The *as2-1 elo4* plant had trumpet-shaped leaves (**Fig. 4E**). The *as2-1 elo4* plants backcrossed twice with Col-0 had somewhat trumpet-shaped or narrow downwardly curling leaves (**Fig. 4F**). These results suggested that the Elongator complex might be required for establishment of the adaxial–abaxial polarity of leaves.

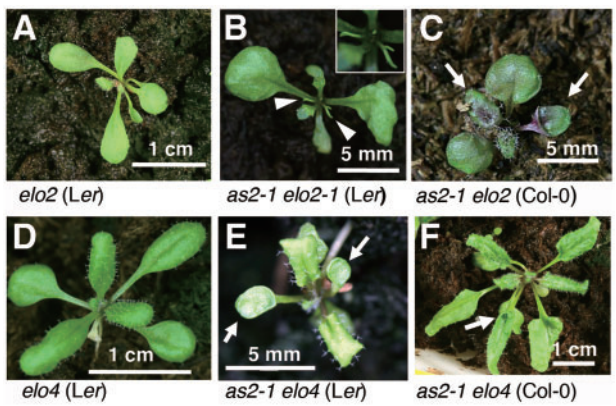

Fig. 4 Mutations in genes for various components of the Elongator complex enhanced adaxial–abaxial defects of *as2-1* leaves. (A) Gross morphology of the *elo2-1* mutant with the L*er* background (L*er*). (B) *as2-1 elo2-1* (L*er*). (C) *as2-1 elo2* with the Col-0 background (Col-0). (D) *elo4* (L*er*). (E) *as2-1 elo4* (L*er*). (F) *as2-1 elo4* (Col-0). All plants shown were photographed 27–28 d after sowing. White arrowheads and arrows indicate filamentous leaves and trumpet-shaped leaves, respectively.

Mutations in *ELO3* and treatment with an HDAC inhibitor caused additive damage to the adaxial development of *as2* leaves

In a previous study, we showed that inhibition of HDACs by trichostatin A (TSA), a specific inhibitor of HDACs, on the *as2-1* background causes severe defects in the adaxial development of leaves, with efficient generation of abaxialized filamentous leaves (Ueno et al. 2007). To investigate the relationship between *ELO3* and genes for HDACs, we treated wild-type (Col-0), *as2-1*, *east1-1* and *as2-1 east1-1* plants with 1 or 3 μM TSA for 2 weeks. In the absence of TSA, wild-type and *as2-1* plants did not produce any filamentous leaves (**Fig. 5A, B**) and the *as2-1 east1-1* double mutant also did not produce any filamentous leaves for 2 weeks (**Fig. 5D**). Upon treatment with 1 μM TSA, wild-type plants exhibited delayed growth but did not produce filamentous leaves (**Fig. 5E**). However, 29% of *as2-1* plants, 4% of *east1-1* plants and 76% of *as2-1 east1-1* plants produced filamentous leaves (**Fig. 5F–I**). Thus, treatment with TSA of *as2-1 east1-1* plants stimulated the production of filamentous leaves in response to the double mutation, as compared with single mutations, and TSA caused the more efficient production of filamentous leaves in *as2-1 east1-1* plants than was observed in the absence of treatment. Wild-type plants treated with 3 μM TSA exhibited significantly delayed growth (**Fig. 5J**), but only 4% generated filamentous leaves. In contrast, 86% of *as2-1* plants, 14% of *east1-1* plants and 28% of *as2-1 east1-1* plants had filamentous leaves after treatment with 3 μM TSA (**Fig. 5K–O**). The lower frequency of filamentous leaves in *as2-1 east1-1* plants at 3 μM TSA might have been due to the severely defective growth of these plants. In fact, 17% of *east1-1* plants and 64% of *as2-1 east1-1* plants failed to develop any true leaves at 3 μM TSA (**Fig. 5M, N, P**). Thus, both treatments with TSA caused marked additional defects in the leaf polarity and growth of *as2-1 east1-1* plants.

Mutations in *ELO3* and *RDR6* or *AGO7* caused additive damage to the adaxial development of *as2* leaves

We investigated the effects of the tasiR-ARF pathway on polarity defects in *east1-1* and *as2-1 east1-1* plants using the *rdr6* and *ago7* mutations. *RDR6* and *AGO7* are involved in the biogenesis of *trans*-acting siRNA, known as tasiR-ARF, that targets *ETT* and *ARF4* mRNAs (Allen et al. 2005, Adenot et al. 2006, Faulgren et al. 2006, Hunter et al. 2006). *rdr6-30* and *ago7-1* mutants have a similar downward-curling leaf phenotype (Vazquez et al. 2004, **Fig. 6D, G**). Our *as2-1 rdr6-30* and *as2-1 ago7-1* plants had narrower cotyledons and more serrated rosette leaves than the wild-type and the *as2* plants, as reported previously (**Fig. 6E, H**; Li et al. 2005, Garcia et al. 2006, Xu et al. 2006). The double mutants *east1-1 rdr6-30* and *east1-1 ago7-1* exhibited a more severe phenotype with more downwardly curling and narrower leaves than the single mutants (**Fig. 6Fa, Ia**). The fifth or sixth leaves often developed two tandem leaf blades on a single petiole (**Fig. 6Fb, Fc, Ib**).

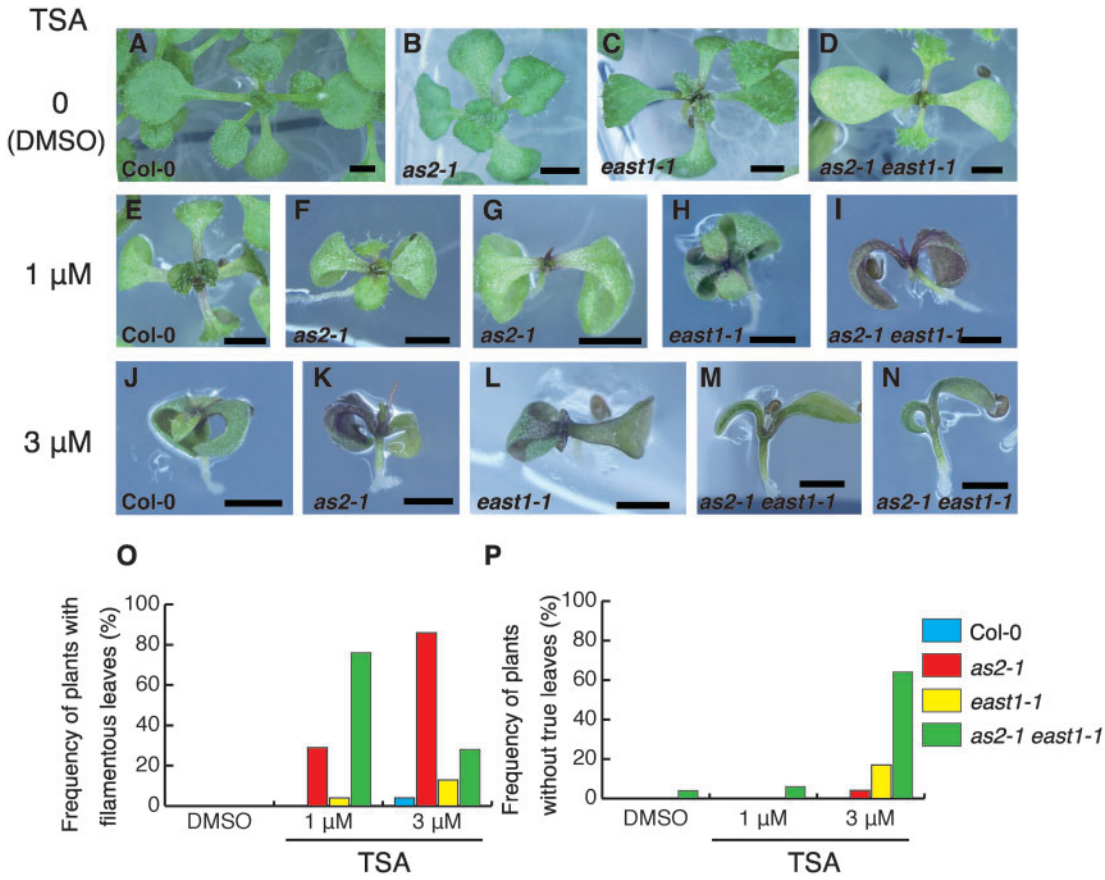

Fig. 5 The HDAC inhibitor TSA caused additional defects in leaf development in *east1-1* and *as2-1 east1-1* plants. Col-0 (A, E, J), *as2-1* (B, F, G, K), *east1-1* (C, H, L) and *as2-1 east1-1* (D, I, M, N) plants were grown for 14 d on MS medium that contained dimethylsulfoxide (DMSO) vehicle (A–D), 1 μM TSA in DMSO (E–I) or 3 μM TSA in DMSO (J–N). The frequencies of plants with filamentous leaves (O) and plants without true leaves (P) were calculated from observations of 49–52 plants for each treatment. Representative data from two experiments, which gave similar results, are shown.

The triple mutant *as2-1 east1-1 rdr6-30* had even more severe defects in leaf development than the *as2-1 east1-1* and *as2-1 rdr6-30* double mutants (**Fig. 6Ja**): plants were significantly reduced in size and developed very narrow cotyledons, only a few leaves with very small leaf blades, and needle-like leaves (**Fig. 6Jb**), but no reproductive organs. The observed leaf phenotype appeared to reflect enhanced defects in the establishment of adaxial–abaxial polarity.

Expression of 34 genes was enhanced in *as1* and *as2* mutants and in plants with mutations in genes for components of the Elongator complex

To identify genes whose expression might be controlled by ELO3, we applied the knowledge-based fuzzy adaptive resonance theory (KB-FuzzyART; Takahashi et al. 2008) to the clustering of profiles of gene expression that were obtained by microarray analysis (with the Affymetrics GeneChip) of mRNAs from wild-type Arabidopsis (*Ler*), *elo3-1*, *elo2-1* and *drl1-2* plants (Nelissen et al. 2005). Since the results in the previous section (**Fig. 4**) indicated that mutations in genes (*drl1*, *elo2* and *elo3*) for various subunits and regulators of the Elongator complex had similar effects on polarity development in *as2* plants, we looked for genes whose levels of expression were altered similarly in the various mutants when compared with wild-type levels. We identified 851 candidate genes. As shown in **Fig. 7A** and **Supplementary Table S1**, we were able to classify these 851 genes by KB-FuzzyArt into clusters I–V, and 45 outliers. These 851 genes did not contain the *AS1* and *AS2* genes.

Clusters III, IV and V included genes whose expression was suppressed in the three mutants. Cluster III included the *IAA13* gene and cluster IV included *IAA3* (*SHY2*), a result that is consistent with the result published by Nelissen et al. (2010). Cluster V included genes, such as *IAA5* and *SAUR*, that are known as auxin-inducible genes. Clusters I and II included genes whose expression was enhanced in the three mutants, and the *STM* gene was categorized to cluster II, consistent with a previously published result (Nelissen et al. 2010). Cluster III included six genes for histones H2A, H2B, H3 and H4, whose

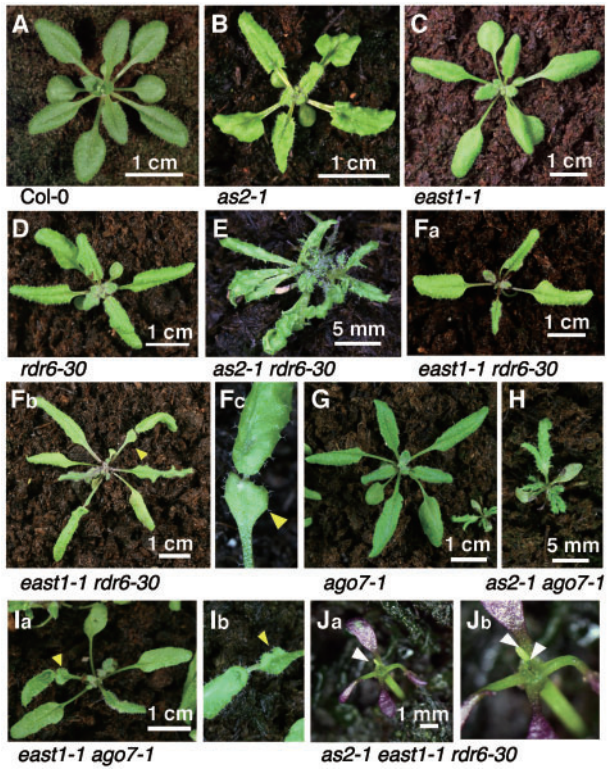

Fig. 6 Introduction of the *rdr6* or the *ago7* mutation into the *east1-1 as2* double mutant enhanced the adaxial defects. (A) Wild type (Col-0). (B) *as2-1*. (C) *east1-1*. (D) *rdr6-30*. (E) *as2-1 rdr6-30*. (Fa) *east1-1 rdr6-30*. (Fb) Thirty-five days after sowing *east1-1 rdr6-30*. (Fc) Magnified view of the rosette leaf in Fb. (G) *ago7-1*. (Ia) *as2-1 ago7-1*. (Ib) Magnified view of the rosette leaf in Ia. (Ja) *as2-1 east1-1 rdr6-30* triple mutant. (Jb) Magnified view of the shoot apex in Ja. Yellow and white arrowheads indicate secondary leaf blades and filamentous leaves, respectively. Plants except those in Fb and Fc were photographed 24 d after sowing.

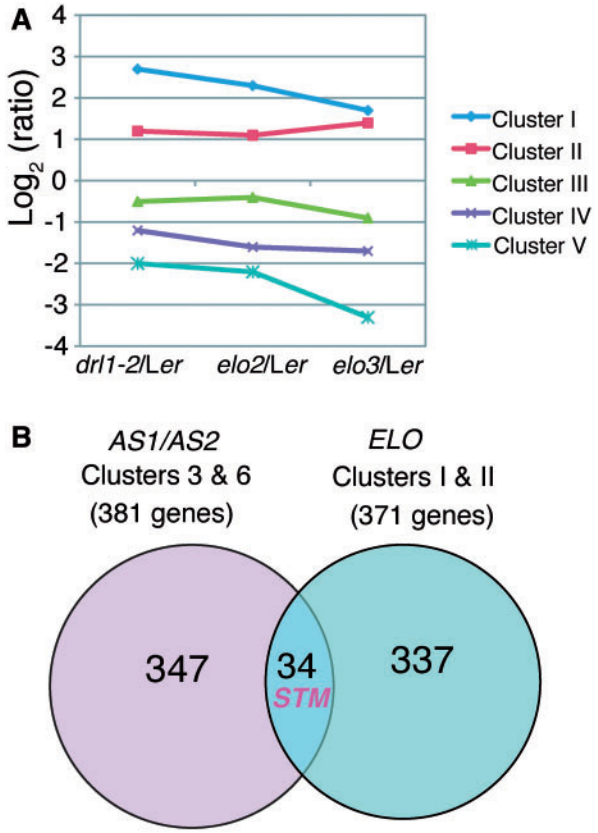

Fig. 7 Mutations of *AS1/AS2* and *ELO3/ELO2/DRL1* both enhanced expression of 34 genes that included *STM*. (A) Representative patterns of expression of genes in each cluster by KB-FuzzyART (see text for details). Signal values for *elo2-1*, *elo3-1* and *drl1-2* plants relative to those for wild-type L*er* are shown with $\log_2$ (ratio). Positive and negative values indicate increased and decreased expression, respectively. (B) Venn diagram of clusters of significantly up-regulated genes in *as1/as2* and in *elo2/elo3/drl*1 plants. The center region indicates genes that appear to be regulated by both ELO3- and AS1/AS2-related pathways.

expression was strongly suppressed in the mutants. It seems that ELO3 might be involved the assembly of chromatin during progression of the S phase.

Clusters I and II (**Fig. 7B**) included 371 genes whose expression was enhanced in *elo3-1*, *elo2-1* and *drl1-2* plants. We previously published the results of cluster analysis of profiles of gene expression in *as2-1* and *as1-1* plants and the overexpressor of *AS2* (Takahashi et al. 2008). Using our new list of genes for cluster analysis (**Supplementary Table S2**), we obtained 18 refined clusters. The *ELO2*, *ELO3* and *DRL1* genes showed no significant changes in microarray data profiles of wild-type, *as2-1* and *as1-1* plants. We identified 381 genes that belonged to two clusters, clusters 3 and 6, whose expression was enhanced both in *as2-1* and in *as1-1* plants and suppressed in the overexpressor of *AS2*. Clusters 3 and 6 included the *BP* and *KAN2* genes, respectively. We observed that expression of 34 genes was enhanced in *as1-*, *as2-* and *elo3*-related mutants (**Fig. 7B**, **Supplementary Table S3**). These genes included *STM*, a meristem-specific gene, as well as *LSH3* and *LSH4*, which are expressed at the boundaries between the shoot apex and leaf primordia (Cho and Zambryski 2011, Takeda et al. 2011).

Discussion

In the present study, we found that *as2-1 east1-1* double-mutant of *A. thaliana* plants formed filamentous and severely serrated leaves with an abaxialized surface (**Figs. 1**, **2**), and our results suggest that the *ELO3* gene is involved in adaxial development on the *as2* background. The *as2* and *elo3/east1-1* mutations resulted in synergistic increases in levels of transcripts of many abaxial-determinant genes, such as *ETT/ARF3*, *FIL* and *YAB5* and all four class 1 *KNOX* genes (**Fig. 2**), some of which have been identified as acting downstream of *AS2* (Semiarti et al. 2001, Iwakawa et al. 2007). Furthermore, our results suggest an essential role for the Elongator complex, which includes

ELO3 and other subunits, in the establishment of adaxial polarity on the *as2* background.

The Elongator complex is involved in repression of abaxial-determinant and class 1 *KNOX* genes through an as yet unidentified pathway that acts in parallel with the AS2/AS1 pathway

There are three possible models to explain how AS2/AS1 and the Elongator complex might be related to each other in the establishment of the adaxial–abaxial polarity of leaves in wild-type *A. thaliana*. In the first model, the Elongator complex might act on adaxialization independently of AS2/AS1 during leaf development: each might regulate common target genes to control the adaxialization of a leaf via two parallel pathways. It has been reported that the AS2/AS1 complex represses transcription of the *BP* and *KNAT2* genes directly (Guo et al. 2008). Transcription of *ETT* is also repressed by *AS1* and *AS2* (Iwakawa et al. 2007). Our expression analysis revealed that levels of *BP*, *KNAT2* and *KNAT6* transcripts were slightly higher in *as2* than in the wild type, consistent with previous results (Semiarti et al. 2001, Iwakawa et al. 2007, Guo et al. 2008) and that the level of the *STM* transcript was slightly elevated in *east1-1* plants and *as1* leaves (**Fig. 2I**; Semiarti et al. 2001). In addition, levels of transcripts of all four class 1 *KNOX* genes were markedly elevated in *as2-1 east1-1* double-mutant plants (**Fig. 2I**). The levels of the transcript of the *FIL* gene was slightly elevated in *as2* or *elo3/east1-1* mutant plants, but markedly elevated in *as2 elo3/east1-1* double-mutant plants. It has been reported that *AS1* represses expression of the *FIL* gene (Garcia et al. 2006). These observations suggest that the Elongator complex might somehow repress the expression of the class 1 *KNOX* and *FIL* genes via an unidentified pathway that might operate in parallel with the AS2/AS1-containing pathway (**Fig. 8**), but we do not yet know whether the Elongator complex controls transcription of these genes directly or indirectly.

In a second possible model, AS2 and the Elongator complex might play biochemically redundant roles in repression of the expression of class1 *KNOX* and abaxial-determinant genes. Since there are no similarities in terms of amino acid sequences between AS2 and ELO3, this possibility seems unlikely.

In a third possible model, AS2/AS1 and the Elongator complex might act via two distinct respective pathways, which might control functionally distinct genes and/or proteins. However, the observed severe defects in the adaxialization of leaf development in the *as2 elo3/east1-1* double mutant might have been due to the synthetic phenotypes of distantly related (or unrelated) phenotypes that might have been generated independently by *as2* and *elo3/east1-1*. Levels of transcripts of class 1 *KNOX* genes, such as *BP* and *STM*, and levels of *FIL* transcripts did, however, increase similarly in the *as2* and *elo3/east1-1* single mutants, and the double mutation clearly raised the levels of transcript of these genes. The similar alterations in patterns of gene expression in *as2* and *elo3/east1-1* single mutants and in the double mutant suggest that this third possibility might be unlikely.

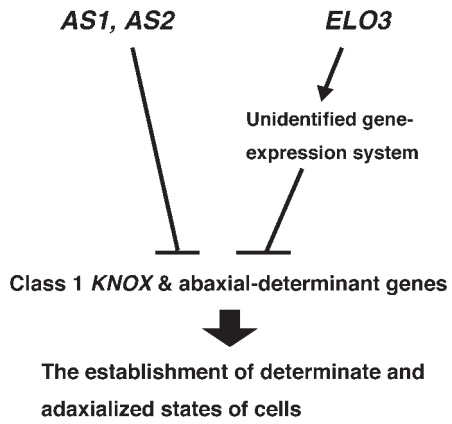

Fig. 8 Proposed models of action of AS2 and ELO3 in repression of the expression of class 1 *KNOX* and abaxial-determinant genes during leaf development. *ELO3* represses the expression of class 1 *KNOX* genes to suppress the indeterminate state in leaf primordia. *ELO3* also represses the expression of genes that are involved in abaxial cell fate on the adaxial side of leaf primordia, presumably acting indirectly, via a distinct pathway that is related to the tasiR-ARF pathway.

The first model provides the simplest explanation of the way in which the AS2/AS1 and the Elongator complex might be involved in the repression of the expression of class 1 *KNOX* and abaxial-determinant genes (**Fig. 8**). In addition, wild-type AS2/AS1 appears to complement potential defects in gene expression generated by mutations in the Elongator complex, as discussed below.

How might the Elongator complex repress expression of abaxial-determinant and class1 *KNOX* genes?

The *ELO3* gene encodes a homolog of yeast Elp3, which has HAT activity, and ELO3, and yeast and mammalian Elp3 proteins are involved in promotion of the transcription of a wide range of genes (Kouskouti and Talianidis 2005, Nelissen et al. 2005, Han et al. 2007, Han et al. 2008, Nelissen et al. 2010). The *elo3-6* mutation of Arabidopsis causes a decrease in levels of acetylation of histone H3K14 in the coding region and 3′-UTRs of the *SHY2/IAA3* and *LAX2* genes (Nelissen et al. 2010), suggesting that histone acetylation by wild-type *ELO3* might allow transcription of these genes. In the present study, the Elongator complex appeared to repress transcription of abaxial-determinant genes and class 1 *KNOX* genes. It is unlikely that the Elongator complex might directly repress such gene expression via histone acetylation. Rather, it is likely that ELO3 might positively control transcription of an unidentified gene whose product might then repress expression of these genes.

In addition to its role in the activation of transcription, yeast Elp3 has been reported to be involved in transcriptional silencing in telomeric regions and in the maintenance of genome

stability through acetylation of histone H3K56 (Li et al. 2009). The Elongator complex also has been shown to play roles in phenomena other than histone acetylation, e.g. modification of wobble uridines in tRNA (Huang et al. 2005, Jablonowski et al. 2006, Mehlgarten et al. 2010), regulation of exocytosis (Rahl et al. 2005), acetylation of α-tubulin (Creppe et al. 2009, Solinger et al. 2010) and demethylation of zygotic parental DNA in mammalian cells (Okada et al. 2010). Although some of these phenomena might be related to gene repression of the type detected in the present study, the mechanisms of repression of the abaxial-determinant and class 1 *KNOX* genes cannot be simply explained by assuming that these events are solely mediated by the Elongator complex or AS2/AS1 alone.

Our cluster analysis identified 34 candidate genes whose levels of expression were enhanced by the *as2*/*as1* and *elo3*/*elo2*/*drl1* mutation. These genes contained not only the *STM* gene, but also two genes, *LSH3* and *LSH4*, which might suppress organ differentiation in the boundary region (Takeda et al. 2011). This result indicates that both AS2/AS1 and the Elongator might promote the determinate state through the repression of these genes. It is worth mentioning that the levels of transcripts of the *STM* genes in *as2 elo3*/*east1-1* mutant plants were greater than those in corresponding single-mutant plants. Recently, Sanmartin et al. (2011) reported that MINIYO (IYO), a positive regulator of transcription, interacts with RNA polymerase II and ELO3. The *IYO* gene functions as a molecular switch for cell differentiation through transcriptional elongation and repression of the *STM* gene. These results support our idea that *ELO3* functions in the establishment of the determinate state.

We observed that later-developing leaves were filamentous or trumpet-shaped leaves in the *as2-1 elo3*/*east1* double mutants, while the cotyledons and juvenile leaves of the double mutants did not show the filamentous or trumpet-shaped phenotype (**Fig. 1**). Recently, Horiguchi et al. (2011) have reported that the *rpl4d-3 as1-1* double mutant shows similar leaf phenotypes. We speculate that some factors involved in the adaxial–abaxial determination in adult leaves may be different from those in cotyledons and juvenile leaves. It might be possible that an unidentified gene expression system, which is regulated by the Elongator, as shown in **Fig. 8**, functions somehow specifically during adult leaf development. We cannot rule out the possibility, however, that the Elongator complex genes themselves are expressed in some specific cells in later stages of leaf development. It might be important to clarify the unidentified system, and further investigation of the molecular functions of AS2 and the Elongator complex will help us to understand these issues during leaf development.

Multiple pathways are involved in the adaxial development of *as2* leaves

In the present study, we showed that *as2 elo3*/*east1-1*, *as2 rdr6* and *as2 ago7* double mutations and treatment of *as2* plants with TSA, an inhibitor of HDACs, caused defects in the establishment of adaxial polarity of leaves. In addition, triple mutations, such as *as2 elo3*/*east1-1 rdr6*, resulted in more severe defects in polarity than double mutations, such as *as2 rdr6*, *as2 ago7* and *as2 elo3*/*east1*. The *RDR6* and *AGO7* genes are involved in the single pathway that is involved in the biogenesis of tasiR-ARF, which is involved in the degradation of *ETT* and *ARF4* transcripts (Allen et al. 2005, Williams et al. 2005, Adenot et al. 2006, Montgomery et al. 2008, Chitwood et al. 2009). Our genetic analysis suggests that the process mediated by ELO3 is distinct from the tasiR-ARF pathway.

Treatment of the *as2 elo3*/*east1-1* double mutant with TSA enhanced the polarity defects of the mutant (**Fig. 5**). Genes for two TSA-sensitive HDACs that are related to the establishment of leaf polarity have been identified as *HDT1* and *HDT2* (Ueno et al. 2007). The observed additive effect of TSA on the polarity defect of the *as2 elo3*/*east1-1* double mutant suggests that HDT1/HDT2 acts independently of the ELO3 pathway. Thus, the adaxial development of leaves might be controlled independently by multiple pathways that include the products of *ELO3*, *RDR6-AGO7* and *HDT1*/*HDT2* on the *as2* background.

Our genetic analysis showed that the wild-type AS2/AS1 complex replaces the polarity establishment function of the *ELO3*, *RDR6* (*AGO7*) and *HDT1*/*HDT2* genes. When *AS2* is functional, adaxial defects due to *east1-1*, *rdr6*/*ago7* and *hdt1*/*hdt2* monogenic mutations are not obvious (**Fig. 6**). Alterations in expression of abaxial-determinant and class 1 *KNOX* genes are also ambiguous in these cases (**Fig. 2**). The adaxialization of leaves is, however, severely disrupted, and expression of abaxial-determinant and class 1 *KNOX* genes is enhanced, once expression of any one of these genes is disrupted in the absence of *AS2* function, even though the molecular events that might be directly governed by these genes (*ELO3*, *RDR6*/*AGO7* and *HDT1*/*HDT2*) do not seem to be closely related to one another.

A wild-type *AS2* gene appears to complement deficiencies in the unrelated events mediated by these latter genes. Further investigations of the molecular functions of AS2 should help us to understand more details of the way in which AS2/AS1 might regulate gene expression.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Col-0 (CS1092), and the *as1-1* (CS3374), *as2-1* (CS3117) and *elo2-3*/*abo1-2* (N504690) mutants were obtained from the Arabidopsis Biological Resource Center (ABRC). We outcrossed *as2-1* with Col-0 three times and *as1-1* with Col-0 once, and used the progeny for our experiments (Semiarti et al. 2001). For analysis of phenotypes, seeds were sown on soil or on plates of MS (Murashige and Skoog) medium. After 2 or 3 d at 4°C in darkness, plants were transferred to a daily regimen of 8 h of darkness and 16 h of

white light at 50 μmol m^{-2} s^{-1} at 22°C, as described previously (Semiarti et al. 2001). Ages of plants are given in terms of numbers of days after sowing. The *elo2-1* and *elo4* mutants were in L*er*, as described (Berná et al. 1999, Nelissen et al. 2005). The seeds of *elo2-1*, *elo4* and *ago7-1* (N537458, available from the ABRC) were kindly provided by Professor J. L. Micol at the Universidad Miguel Hernández and Professor Y. Watanabe at the University of Tokyo.

Screening of enhancer of *as2-1*

About 2,000 *as2-1* seeds were treated with 0.4% ethylmethane sulfonate (EMS) for 8 h and then these M_1 seeds were germinated on soil. M_2 seeds derived from 13–33 M_1 plants were collected to obtain the 81 stocks of M_2 seeds. At least 100 plants from each stock were grown on the soil or MS agar medium, and the leaves were observed for morphology. Each enhancer mutant was crossed with wild-type Col-0 to generate F_1 seeds. F_2 seeds were grown on the soil and about 5% of the progeny showed a phenotype similar to the original phenotype. We also isolated the *east4* mutant, which was a new *rdr6* mutant allele, namely *rdr6-30*, from the population of M_2 seeds (our unpublished data).

Map-based cloning of *east1-1*

The F_2 population of a cross between *east1-1* and *as2-5* (L*er*) was used for the genetic mapping of *east1* with mapping primers as described previously (Tanaka et al. 2004). For complementation, 5.7 kb of a *Sac*I–*Bal*I genomic fragment was cloned into the binary vector pGreen0029 (Hellens et al. 2000) and transformed into *as2-1 east1-1* plants by *Agrobacterium*-mediated transformation (Bechtold and Pelletier 1998). A kanamycin-resistant T_1 plant with *as2-1* phenotype was isolated and T_2 progeny were segregated to kanamycin-sensitive and kanamycin-resistant populations with *as2-1 east1-1* and *as2-1* phenotype, respectively. Primers for high-resolution mapping and genotyping of the mutations are listed in **Supplementary Table S4**.

Real-time RT–PCR

Leaves and shoot apices of wild-type and mutant plants were harvested at 14 d after sowing, and immediately frozen in liquid nitrogen and stored at −80°C. Total RNA was isolated by using the RNeasy Plant Mini Kit (Qiagen). For the analysis of RNA levels by real-time PCR, we prepared 5 μg of total RNA to convert to cDNA as described by Iwakawa et al. (2007). PCR was performed in the presence of the double-stranded DNA-specific dye *Power* SYBR Green (Applied Biosystems) with primers as described (Iwakawa et al. 2007). Amplification was monitored in real time by using the Applied Biosystems StepOnePlus Real-Time PCR system, according to the supplier's recommendations. The mean value of three technical replicates was normalized by the value of *ACTIN2* transcripts.

Fluorescence microscopy

We crossed *as2-1 east1-1* and *as2-1 FILp:GFP* (Watanabe and Okada 2003, Ueno et al. 2007). Shoot apices containing leaf primordia were embedded in 7% agarose, and agarose blocks were sliced into thin sections. Fluorescence was observed with an LSM510 confocal laser scanning microscope (Carl Zeiss Inc.).

Microarray data analysis of *as1-1*, *as2-1* and *as2-1/pAS1::AS2* mutants

Gene expression data were obtained by using ATH1 Affymetrix chips (Affymetrix) and total RNA from shoot apices of 15-day-old Col-0, *as1-1*, *as2-1* and *as2-1*/p*AS1::AS2* plants as described (Takahashi et al. 2008). In this study, we conducted reanalysis using new list of genes for *A. thaliana* (**Supplementary Table S2**). A total of 18 clusters were constructed for the same array data set by KB-FuzzyART (Takahashi et al. 2008). Clusters 3 and 6 among these clusters were merged and used in the following analysis, as shown in **Supplementary Table S3**.

Microarray data analysis of *elo2-1*, *elo3-1* and *drl1-2* mutants

The Affymetrix microarray data for L*er*, *elo2-1*, *elo3-1* and *drl1-2* mutants (Nelissen et al. 2005) were retrieved from ArrayExpress (E-MEXP-300). Data were obtained from a total of 12 arrays (triplicate experiments for four strains). The raw data were processed by using Affymetrix Gene Chip Operating Software (GCOS; Version 1.4.0.036). Signal intensities were calculated by using perfect match/mismatch probe pairs and scaled to a target intensity of 200 by using GCOS. For these data, it is possible to calculate nine pairs (triplicate experiments of mutant)/(triplicate experiments of wild type) for each set of mutant/wild-type ratios. Therefore, a total of 27 signal ratio data points (9 pairs × 3 mutants) were calculated by using GCOS. The detection calls and change calls were calculated based on the signal intensity ratio between perfect match and mismatch oligos on the array. The detection calls are a present, marginal or absent call (i.e. detection call determined by GCOS, based on the *P*-value of the one-sided Wilcoxon signed-rank test, present call means $0 \leq P < 0.05$, marginal call means $0.05 \leq P < 0.065$ and absent call means $0.065 < P \leq 1$, respectively). The change calls are an increase, no change or decrease call (i.e. change call determined by GCOS, based on the *P*-value of the one-sided Wilcoxon signed-rank test, increase call means $0 \leq P < 0.006$, no change call means $0.006 \leq P \leq (1-0.006)$ and decrease call means $(1-0.006) < P \leq 1$, respectively). In the present study, we used 22,591 genes (excluding probe sets for control and mitochondria) from 22,810 genes on the ATH1 chip. For gene selection, we excluded 5,940 genes for which all 12 samples, showed an absent call, and 7,347 genes for which all 27 pairs showed no change calls. Therefore, we included those genes (6,888 genes)

for which all calls showed the same pattern of calls for each mutant, because the same pattern of calls indicates high reproducibility. Finally, 851 genes were selected and the median signal ratios for each gene were calculated from nine pairs of ratios for each set of mutant/wild type. These data were used for clustering analysis.

Supplementary data

Supplementary data are available at PCP online.

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